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AGROLABO Filarcheck ELISA

ELISA Test for the detection of *Dirofilaria immitis*
In dog serum or plasma

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1. INTRODUCTION

Cardiopulmonary filariasis is an emininct infection with a cosmopolitan geographical distribution: the parasite, in fact, can be found in all continents, although its prevalence varies strongly.

Dogs are considered the definitive host for heartworms (*Dirofilaria immitis*). However, heartworms may infect more than 30 species of animals (e.g., coyotes, foxes, wolves and other wild canids, domestic cats and wild felids, ferrets, sea lions, etc.) in addition to humans as well.

Filarial nematodes described in dogs are: *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum, A. dracunculoides* and *Cercopitifilaria grassi* (Order: Spirurida, Superfamily: Filarioidea, Family: Onchocercidae). The most prevalent species are *D. immitis, D. repens,* and *A. reconditum,* that show a different geographical distribution: cosmopolitan for *A. reconditum* and *D. immitis,* restricted to the Europe, Middle East, Asia and Africa for *D. repens.* *D. immitis* is responsible for heartworm disease, whereas the other species produce subcutaneous or splanchnic infections. Furthermore, in areas where dog filariasis are endemic, at least *D. immitis* and *D. repens* are recognized as etiological agent of zoonotic infections in humans.

1.1 *Dirofilaria immitis* Life Cycle

When a mosquito carrying infective heartworm larvae bites a dog, it transmits the infection. The larvae are able dwell in the derma for 3-6 days, after which they migrate to the haematic and linfatic capillaries, followed by the heart and the pulmonary artery, where they develop into sexually mature adults. The worms then mate and the females release their offspring (microfilariae) into the blood stream.

The time elapsed from when the larvae enter the dog until the minute offspring can be detected in the blood (pre-patent period) is about six to seven months. The male heartworms (four to six inches in length) and the females (10-12 inches) become fully grown about one year after infection, and their life span in dogs appears to be at least five to seven years.

In experimentally induced infections of heartworms in dogs, the percentage of infective larvae developing to adults is high (40% to 90%). However, the percentage of experimentally infected dogs from which adult worms are recovered is virtually 100%. The number of worms infecting a dog is usually high, as the number of worms in dogs can range from one to approximately 250.

Microfilaremia, the presence of heartworm offspring in the blood of the host, is relatively common in dogs. However, not all heartworm infections result in such offspring circulating in the blood. These are known as occult heartworm infections and may be the result of a number of factors such as single sex heartworm infections, host immune responses affecting the presence of circulating offspring (microfilariae) and most significantly, the administration of heartworm preventives.
1.2 Canine infections

The onset and severity of disease in the dog is mainly a reflection of the number of adult heartworms present, the duration of the infection, and the level of activity of the dog. Dogs with higher numbers of worms are generally found to have more severe heart and lung disease changes. Until the number of mature heartworms exceeds 50 in a 25-kg dog (approximately 55 pounds), nearly all of the heartworms reside in the lower caudal pulmonary arteries (the arteries of the lower lung lobes). Higher numbers of heartworms will result in their presence in the right chambers of the heart. In such infections, the most common early pathological changes caused by heartworms are due to inflammatory processes that occur in and around the arteries of the lower portion of the lungs in response to the presence of heartworms. Later, the heart may enlarge and become weakened due to an increased workload and congestive heart failure may occur. A very active dog (e.g., working dog) is more likely to develop severe disease with a relatively small number of heartworms than an inactive one (e.g., a lap dog or couch potato). In an occasional dog with a large number of heartworms, the worms may not only be in the heart but also the caudal vena cava (large primary vein of the lower body) between the liver and the heart. This syndrome (Vena Cava or Liver Failure Syndrome) is characterized by sudden collapse and even death within two to three days if they are not removed surgically.

1.3 Clinical Signs

Heartworm disease may cause combinations of medical problems within the same dog including dysfunction of the lungs, heart, liver and kidneys. The disease may have an acute onset but usually begins with slow barely detectable signs resulting from a chronic infection with a combination of physiologic changes. Dogs with a low number of adult worms in the body that are not exercised strenuously may never have overt signs of heartworm disease. The heart and lungs are the major organs affected by heartworms in dogs with varying degrees of clinical signs.

1.4 Diagnosis

The diagnosis of filariasis in dogs is usually performed by the visualization of microfilariae in blood smears, or by the detection of specific antigens released by adult or immature parasites. The decision to adopt either one or the other method may lead, in some cases, to diagnostic failures: although more rarely, a positive blood smear may be associated with a negative serological response, while in most animals with a negative blood smear the application of a serologic protocol reveals the presence of circulating antigens (Table 1).

<table>
<thead>
<tr>
<th>Microfilaraemia</th>
<th>Serologic test</th>
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</thead>
<tbody>
<tr>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>POS</td>
<td>NEG</td>
</tr>
<tr>
<td>(low parasitic load)</td>
<td></td>
</tr>
<tr>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>(occult filariasis)</td>
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<tr>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>(occult filariasis)</td>
<td>(low parasitic load)</td>
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Table 1 – Diagnostic options in dogs with filariasis.
The first possibility is related to the sensitivity of the serological tests adopted by the veterinarian, which is dependent on the parasitic load. In the second case, the serologic tests represent the only laboratory tests adequate to confirm a clinical suspect.

The progressive increase of occult parasitic infections as a consequence of the use of filaricidal and microfilaricidal drugs both for prophylactic and therapeutic purposes, has lead to a re-evaluation of the serologic diagnosis for this disease.

The so-called “occult phase” of the disease can be detected through antigen detection. Occult heartworm infections can be the result of a number of factors. Single sex heartworm infections, host immune responses affecting the presence of circulating microfilariae and the administration of heartworm preventives can be factors which produce occult infections in dogs. Antigen detection is also an important tool for the evaluation of the effectiveness of microfilaricide treatment. In fact, negative results of antigen testing carried out following an adequate lapse of time after therapy (generally 3 months post-treatment), provide objective proof of the effectiveness of treatment.

Moreover, the serologic test is of paramount importance both in cases of immunomediated occult filariosis (where the immunological response of the organism is able itself to clear the microfilariae for peripheral blood), and in the evaluation of the efficacy of the adulticide treatment with specific drugs, by the detection of a post-treatment negative serologic test. In these circumstances the antigens released by the parasites disappear from peripheral blood usually 3 months following a successful treatment.

Last but not least, the adoption of a serologic test able to detect with adequate sensitivity the presence and the parasitic load, may help for a better classification of the stage of the disease, according to clinical symptoms and treatment decisions.

The diagnostic steps must therefore include the detection of microfilariae in peripheral blood smears (especially in those areas where the parasitic load may be low), followed by a serologic investigation aimed to detect, with the most sensitive and specific test commercially available, the presence of circulating antigens, with the aim to evaluate the best options for therapy and the subsequent follow-up of the treated animals.

Therefore, the need for highly sensitive and specific tests is today even more perceived by the veterinarian, which additionally requires a quick, simple, single and “on the bench” test.

The commercially available tests must be evaluated for their specificity (i.e. their ability to specifically detect antigens secreted by *Dirofilaria immitis* and excluding positives for *D. repens*) and sensitivity (i.e. their capacity to detect the minimal available amount of *D. immitis* circulating antigen). For the choice of the test to
be used, other considerations are related to the area where the investigations are to be performed: the serological tests will be widely used in the regions where filariasis is endemic, while in areas characterized by low prevalence of the disease the antigen detection test will be primarily applied in animals with occult filariasis.

2. PRINCIPLE OF TEST

The FILARCHEK test is based on a sandwich ELISA (Enzyme Linked Immunosorbent Assay) technique. The microplate wells are coated with a monoclonal antibody against the circulating antigen of *D. immitis*. Canine serum is added into the wells. If the serum contains the antigen, an antigen-antibody complex (Ag-Ab) will form and fix on the solid phase.

During the second phase a peroxidase conjugated antibody (Ab*) reacting to a different antigenic epitope, is added and binds to the Ag-Ab complex. After washing, the chromogen substrate is added. This will react and turn visibly blue when the Ag-Ab-Ab* complex is present. If the canine serum does not contain the antigen, the Ag-Ab complex will not form, and serum components will be flushed out with washing.

3. MANUFACTURING OF THE FILARCHECK ELISA KIT

The production protocol of FILARCHEK includes the following steps:

1. Sensitization of ELISA plates.
2. Preparation of reagents.
3. Quality Control.

3.1 Preparation of ELISA plates

- The polistiren ELISA plates are sensitized with a highly purified monoclonal antibody against specific epitophies of the cuticolar antigen of *D. immitis*.
- The plates are treated by a process of saturation, in order to eliminate any aspecific binding present on plate.

3.2 Preparation of reagents

- The secondary antibody is an anti-cuticular antigen of *D.immitis* that is conjugated with peroxidase.
- It is supplied ready to use, and is diluted in a solution that contains a preservative which helps maintain its stability.
- The negative control is a non infected dog serum deriving from non endemic Filariasis areas. The control blood is tested for infections from other parasites through approved parasitological and serological methods.
- The positive control is a Filariasis infected dog serum, that is proved positive by both parasitological and serological analysis.

3.3 Quality Control

- Each batch of ELISA FILARCHECK is accurately checked to evaluate the proper functioning of all reagents, batch homogeneity (intra and inter-plate repeatability), and the integrity kit components (droppers, bottles, pipettes).

4. TEST VALIDATION

Using a panel of serum with characteristics of filariasis, a comparative study between ELISA FILARCHEK and Dirocheck ELISA (Synbiotics) was performed. The Dirocheck ELISA test was used as a reference test (golden standard).

4.1 Samples

107 serum or plasma samples were selected from naturally infected or healthy subjects. All of the serum samples were analyzed in parallel in order to compare the two tests.

4.2 ELISA Dirocheck

This immunoenzymatic test is based on the same principle of Filarcheck test. The test was performed following the instructions supplied with the kit.

The samples were added to the plate and, after the addition of the conjugate, were incubated for 10 minutes at room temperature (RT). After washing to eliminate any unbound materials, the chromogen-substrate of peroxidase enzyme was added. The substrate determines the development of a blue colour for positive samples, while negative samples remain uncoloured.

The interpretation of results was carried out visually, comparing the blue colour of samples with the colour of the negative and positive controls.

4.3 ELISA Filarcheck

The Filarcheck test was performed on all 107 samples using the protocol described in the kit instruction insert.

The samples were undiluted and incubated for 10 minutes at RT, with the appropriate amount of conjugate. Following washing to eliminate any unbound materials, the plate was incubated at RT for a further 10 min
with the addition of a chromogen substrate. The peroxidase enzyme substrate determines the development of a blue colour when samples are positive, while negative samples remain colourless. The interpretation of results was carried out visually, by comparing the blue colour of samples with the colour of the negative and positive controls.

5. TEST VALIDATION RESULTS

5.1 Performance of ELISA FILARCHECK test

The sensitivity (Se) and specificity (Sp) and the agreement (K) values of FILARCHECK test versus Dirocheck test were calculated. These parameters were calculated using the following formula:

\[
\begin{align*}
\text{Sp} & = \frac{\text{VN FIL}}{\text{Neg DIR}} \times 100 \\
\text{Se} & = \frac{\text{VP FIL}}{\text{Pos DIR}} \times 100 \\
\text{K} & = \frac{\text{VN FIL} + \text{VP FIL}}{\text{Total sera}} \times 100
\end{align*}
\]

Neg DIR: number of negative samples with DIROCHECK;
Pos DIR: number of positive samples with DIROCHECK;
VN FIL: number of true negative samples with FILARCHHECK;
VP FIL: number of true positive samples with FILARCHCHECK;
FN FIL: number of false negative samples with FILARCHCHECK;
FP FIL: number of false positive samples with FILARCHCHECK.

The 107 analyzed sera were classified with Dirocheck as 66 negatives and 41 positives. The Filarcheck test correctly identified 40 positive samples out of 41, and 66 negative samples out of 66, showing a sensitivity of 97.6% and specificity of 100% (Tab. 3). The agreement with Dirocheck was equal to 99.1%.

<table>
<thead>
<tr>
<th>DIROCHECK</th>
<th>FILARCHCHECK</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS</td>
<td>NEG</td>
</tr>
<tr>
<td>POS</td>
<td>40</td>
</tr>
<tr>
<td>NEG</td>
<td>0</td>
</tr>
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</table>

Tab.3: Comparison of the FILARCHCHECK test with the DIROCHECK test on a 107 dog sera panel

Sensitivity (Se) = 97.6 %
Specificity (Sp) = 100 %
Agreement (K) = 99.1 %